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Regular Article

# Simvastatin attenuates the endothelial pro-thrombotic shift in saphenous vein grafts induced by Advanced glycation endproducts

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ABSTRACT

*Background:* Advanced glycation endproducts (AGEs) and its receptors (RAGEs) are heterogeneous signaling proteins associated to diabetes and responsible of endothelial alterations leading to atherosclerosis progression and graft failure. The aim of this study was to investigate the role of statin in reducing AGEs related endothelial damage.
*Methods:* Endothelial cell(EC) obtained from leftovers of saphenous vein grafts of non-diabetic patients were incubated with AGEs (2 and 20 μM) and subsequently treated with Simvastatin. Neutrophils (PNM) adherence, ROS production and RAGE and peroxisome proliferator-activated receptors-gamma (PPAR-γ) expression were analyzed. As clinical validation of the in vitro findings, ECs of diabetic patients in optimized glycaemic control administered with a 3 weeks Simvastatin regimen were similarly processed.
*Results:* Simvastatin blunted the rise in PMN adhesion and ROS generation following stimulation of saphenous vein EC culture with AGEs in vitro. This effect was time dependent and was associated to an increase in *PPAR-γ* induction paralleled by a decrease in RAGEs expression. Parallely, data from diabetic patients administered with Simvastatin showed a similar significant reduction in PNM adhesion and ROS generation. Simvastatin treatment significantly decreased RAGEs expression in ECs from diabetic patients and determined a slight increase in PPAR-γ expression but the latter failed to reach statistical significance. Interference in the function of these two crucial pathways might be at the root of the statin antinflammatory and antithrombotic effect in the context of AGEs-associated damage.
*Conclusions:* Despite the recently raised warning on the use of statins in the diabetic population, this study elucidates their cornerstone position in endothelial homeostasis of saphenous grafts in patients with controlled diabetes.


## Introduction

Increasing evidences are pointing at advanced glycation endproducts (AGEs) and its receptors (RAGE) as the responsible of a large spectrum of molecular alterations eventually leading to plaque progression and complications [1] but also to graft failure [2] or restenosis after cardiovascular surgical procedures in diabetic patients [3]. In the clinical context, the latter achieves a particular significance considering the increasing number of interventions performed on diabetics and the possible therapeutic scenarios that a pharmacologic approach oriented against AGEs-RAGE axis might open. We recently demonstrated *in vitro* that the presence of a large AGE burden in the vessel wall increases the likelihood of an exaggerated and prolonged inflammatory reaction and determines a pro-thrombotic state, defining a common mechanism potentially explaining the increased rate of vein graft failure after coronary bypass surgery. In vitro stimulation of saphenous vein endothelial cells with AGEs determined i) an upregulation of endothelial RAGE expression, ii)a dose dependent increment of polymorphonucleates (PMN)-endothelium adhesion iii) increased production of reactive oxygen species (ROS) from endothelial cells (ECs) and iv) decreased endothelial PPAR-γ expression [2]. On account of the reported pleiotropic effects of HGMCoA-reductase inhibitors and of their mitigating action on AGE-RAGE-mediated damage [3], we followed our previous set of

*Abbreviations:* CABG, Coronary Artery Bypass Grafting; AGEs, Advanced Glycation Endproducts; RAGEs, Receptors of Advanced Glycation Endproducts; PPAR-γ, peroxisome proliferator-activated receptors-gamma; DM, Diabetes Mellitus; ROS, Reactive Oxygen Species; PNM, polymorphonucleates; EC, Endothelial Cells; BSA, Bovine Serum Albumine; DCF, 2′,7′-dichlorofluorescein.

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experiments investigating the effect of Simvastatin in AGEs-induced endothelial activation including PMN adhesion, ROS production, RAGEs expression and PPAR-γ induction.

## Methods

### *Study design*

This study was conducted on saphenous vein grafts leftovers obtained from 30 non-diabetic patients scheduled for coronary surgery. Patients enrolled have no evidence of systemic inflammatory diseases, malignancies, hematopoietic disorders, renal failure, leg vein insufficiency and were not administered with statin or PPAR-γ agonist (i.e. thiazolidinediones) and/or steroids within the last 6 months. Segments of saphenous vein grafts were *ex vivo* cultured in presence of AGEs for 24 hours and then treated with Simvastatin for 24 hours to perform leukocyte adhesion assay. Remaining segments were used to isolate and culture ECs which have been further stimulated with AGEs, similarly treated with statin and eventually assayed for ROS production, RAGEs expression and PPAR-gamma induction. In parallel to our previous study, we performed an additional set of experiments with diabetic patients for validation purposes. Thirty type 2 diabetic patients under optimal glycaemic control (HbA1c level < 6.0%), scheduled for coronary surgery were also enrolled (demographics in Table 1). Exclusion criteria were represented by non-optimized glycemic control (HbA1c level > 6.0%), proliferating retinopathy, peripheral neuropathy, renal failure, leg vein insufficiency, any therapy with PPAR-γ agonist (i.e. thiazolidinediones or statin) and/or steroid therapy within the last 6 months. AGEs serum levels were measured and patient exhibiting values > 7 μg/ml were included [2]. Accordingly to the literature and our previous report, this value is associated to diabetic state and to diabetes-induced damage to endothelium of cardiac vessels [4,5] and microvasculature [6]. Routine biochemical markers, Hb1Ac, inflammatory parameters and oxidative stress markers, including serum malondialdehyde (MDA) [7] and oxidized low density lipoprotein (oxLDL) [8], were measured and the values reported in Table 1. Fifteen patients received 20 mg/day Simvastatin for 3 weeks before surgery. Venous conduits were harvested and endothelium isolated and further processed similarly to the principal study group. In order to estimate the total number of patients and samples required to demonstrate the study outcomes an inverse power analysis was performed. Data previously generated on the modulation of neutrophil-endothelium interaction on saphenous vein of patients undergoing coronary bypass grafting [9] and on RAGEs and PPAR-γ expression induction [2] were used in the calculation. The study conforms to the Declaration of Helsinki. The Local Ethical Committee approved the protocol, and all individuals provided informed consent.

**Table 1**
Demographic data and laboratory findings including AGEs and Hb1AC levels, inflammatory parameters and oxidative stress markers of both the control and the diabetic population.

| Parameter | Control (N 30) | Diabetes (N 30) | P value |
|---|---|---|---|
| Age (yrs) | 68,75 ± 6,54 | 66,91 ± 10,1 | 0,78 |
| Male gender (%) | 23 (76,7%) | 22 (73,3%) | 0,45 |
| Hypertension (%) | 28 (93,3%) | 26(86,6%) | 0,57 |
| Dislipidemia (%) | 25 (83,3%) | 28 (93,3%) | 0,48 |
| Smoke (%) | 14 (46,6%) | 11 (36,6%) | 0,37 |
| Family History of CVD | 21 (70%) | 23 (76,6%) | 0,87 |
| Peripheral vascular disease | 18 (60%) | 20 (66,6%) | 0,52 |
| Recent AMI | 11(36,6%) | 12 (40%) | 0,47 |
| Medications | | | |
| • ACE-inhibitors assumption | 26 (86,6%) | 24 (80%) | 0,46 |
| • B-Blockers | 23 (76,6%) | 21 (70%) | 0,86 |
| • Calcium antagonists | 16 (53,3%) | 19 (63,3%) | 0,27 |
| • Diuretics | 8 (26,6%) | 9 (30%) | 0,64 |
| WBC (cells/μl) | 6,546 ± 4,372 | 7,289 ± 2,897 | 0.47 |
| Hb (g/dL) | 12.3 ± 2,1 | 11.4 ± 1.4 | 0.42 |
| AST (IU/L) | 72.4 ± 21,48 | 75,98 ± 37,45 | 0.59 |
| ALT (IU/L) | 30.2 ± 11,38 | 31,8 ± 18,79 | 0.65 |
| γ-GTP (IU/L) | 31.9 ± 13.7 | 34.5 ± 12,32 | 0.97 |
| Total bilirubin (mg/dL) | 0.9 ± 0.2 | 1,02 ± 0.7 | 0.47 |
| BUN (mg/dL) | 12.6 ± 3,2 | 13,8 ± 2.8 | 0.84 |
| Creatinine (mg/dL) | 0.94 ± 0.33 | 0.92 ± 0.49 | 0.61 |
| Uric acid (mg/dL) | 4,1 ± 1.9 | 4.9 ± 2.3 | 0.28 |
| TC (mg/dL) | 243.5 ± 28.2 | 241.9 ± 32.7 | 0.46 |
| LDL-C (mg/dL) | 147.8 ± 27.4 | 143.1 ± 31.7 | 0.79 |
| TG (mg/dL) | 112.6 ± 49.3 | 114.8 ± 51.6 | 0.47 |
| HDL-C (mg/dL) | 43.7 ± 11.8 | 44.1 ± 11.2 | 0.81 |
| hs-CRP(mg/L) | 23.7 ± 26,8 | 30.1 ± 19.7 | 0.78 |
| HbA1c (%) | 4.3 ± 1.7 | 5.1 ± 0,8 | 0.023 |
| AGEs (μg/ml) | 2.3 ± 1,2 | 9.1 ± 3.9 | 0.04 |
| Malondialdheide MDA (μmol/L) | 2,82 ± 0,7 | 3,27 ± 1,4 | 0.87 |
| Ox-LDL (ng/ml) | 91.82 ± 5,98 | 93.31 ± 4.12 | 0.42 |

Abbreviations
WBC, white blood cell counts; Hb, hemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-guanosine 5'-triphosphate; BUN, blood urea nitrogen; TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; HbA1c, haemoglobin A1c (glycated haemoglobin); AGEs, Advanced glycation end products AGE; Ox-LDL, oxidized LDL.

### *Preparation of AGE – BSA complexes (AGEs)*

The glycated Bovine Serum Albumine (BSA) was prepared according to the method of Horiuchi et al. [10]. with minor modifications. The AGE-BSA complex was characterized by mobility in sodium dodecyl sulphate-polyacrylamide gel electrophoresis, absorption and fluorescent spectra (370–440 nm).

### *Saphenous vein harvesting, AGE-mediated activation and statin treatment*

Great saphenous vein was harvested by a no touch technique, stored in heparinized blood and immediately employed for grafting. Discarded vein segments were immediately stored in sterile M 199 culture medium in humidified incubator at 37 °C and 5% $CO_2$. Segments were then incubated with AGE at concentrations of both 2 μM and 20 μM and control media represented by no-glycated BSA in order to avoid biases concerning non-specific effect of BSA [11]. After 24 hours of culturing 5μM Simvastatin (Merck Sharp&Dohme, Whitehouse Station, NJ) was added and segments collected after 24 hours for further adhesion assay.

### *Neutrophil isolation and adhesion assay*

Blood sample were collected by venipuncture from patients and neutrophils were isolated by Ficoll-Hypaque density gradient centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes. Adhesion assay following fluorescent labeling of isolated neutrophils was performed as previously described [9]. Number of neutrophils adhering to the endothelial surface in five separate microscopic fields were counted manually on a microscope equipped for fluorescence, using the filter IF355–550.

### *Endothelial cell cultures, AGE-mediated activation and statin treatment*

Human ECs were isolated from segments of saphenous veins and cultured as previously described [2]. All the experiments were performed on ECs at passages 2 to 5. Similarly to vein segments, ECs were cultured in presence of AGE at concentrations of 2 μM and 20 μM or control medium containing non-glycated BSA for 24 hours and subsequently cultures were added with 5μM Simvastatin (Merck Sharp&Dohme, Whitehouse Station, NJ).

### *Endothelial cell RNA Extraction and Reverse Transcription (RT)-PCR*

Total RNA was extracted from cultured endothelial cells using TRI Reagent (Sigma Aldrich), as described by Chomczynski and cDNA obtained [12]. Amplification of PPARγ and RAGE cDNA was performed as